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(54) Title: METHOD OF AFFINITY CROSS-LINKING BIOLOGICALLY ACTIVE, IMMUNOGENIC PEPTIDES TO ANTIBODIES

(57) Abstract

A method of affinity cross-linking a peptide to an antibody by photo-chemically activating an azido compound in a peptide comprising said azido compound; adding an antibody to the photochemically activated peptide; and allowing the photochemically activated peptide and the antibody to react. The azido compound has an affinity for a hydrophobic structure in the variable domain of the antibody which binds to nucleotides or nucleosides, binding the peptide into a native binding pocket of the immunoglobulin (Ig) structure of an antibody. The site of cross-linking is located away from the antigen binding site in the Fv domain avoiding the compromise of antigen recognition. A composition of a peptide cross-linked to an antibody is also disclosed.

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METHOD OF AFFINITY CROSS-LINKING BIOLOGICALLY ACTIVE, IMMUNOGENIC PEPTIDES TO ANTIBODIES

FIELD OF THE INVENTION

The present invention relates to a method of chemical cross-linking biologically active peptides to antibodies and a composition comprising one or more peptides cross-linked to an antibody. Specifically, the method attaches peptides to a unique affinity site of antibodies using photoreactive derivatives of peptides. These antibody-peptide complexes have immunostimulatory properties for B-cells and T-cells as a molecular adjuvant conjugated antibodies or antibody fragments. In addition the complexes may have biological properties to enhance antibody binding, facilitate membrane transport of antibodies or their fragments, and present B and T-cell epitopes to antigen presenting cells.

BACKGROUND OF THE INVENTION

Antibodies have been praised as "magic bullets" to combat disease. However, the promises made for antibodies were never fully realized. This is in part due the fact that antibodies represent only one arm of the immune defense, where T-cells provide the other strategy in immune defense. However, antibodies are ideal targeting and delivery devices. They are adapted for long survival in blood, have sites which help vascular and tissue penetration and are functionally linked with a number of defense mechanisms of the innate immunity. One such mechanism is the complement system which helps to destroy pathogens and is involved in the regulation of immune responses. For example the complement fragment C3d binds to the CR2 receptor on B-cells, which is also the binding site for Epstein-Barr virus. Binding of Epstein-Barr virus to CR2 activates B-cells. Accumulated evidence has shown that the CR2 receptor (CD19/Cd20/CD81 complex) has an immuno-stimulatory role and is activated by C3d.

Another example of how antibodies can be used to enhance the immune response has been demonstrated by the work of Zanetti and Bona (Zanetti, M. Nature 355: 466-477, 1992;

Zaghouani H.; Anderson S.A., Sperbeer K.E., Daian C. Kennedy R.C., Mayer L. and Bona C.A. 1995 Proc. Nat. Acad. Science U.S. 92: 631-635). These authors have replaced the CDR3 sequence of the Ig heavy chain with a sequence resembling T-cell and B-cell antigens (epitopes) using molecular biology methods and have shown that these modified antibodies induce potent immune response specific for the inserted groups. While this method of CDR replacement or antigenizing is effective, it requires manipulation of the genes encoding the immunoglobulin chains and expression of the modified antibodies with fermentation methods, both of which are expensive and time consuming.

THe biological properties of the antibodies can be enhanced with respect to overall avidity for antigen and the ability to penetrate cellular and nuclear membranes. Antigen binding is enhanced by increasing the valency of antibodies such as in pentameric IgM antibodies. Valency and avidity is also increased in certain antibodies which are self binding or homophilic (Kang, C.Y., Cheng, H.L., Rudikoff, s. and Kohler, H. J. Exp. Med. 165:1332, 1987). Xiyun, A.N., Evans, S.V., Kaminki, M.J., fillies, S. F.D., Resifeld, R.A., Noughton, A.N. and Chapman, P.B. J. Immunol. 157: 1582-1588 (1996)). A peptide in the heavy chain variable region was identified which inhibited self-binding (Kang, C.Y. Brunck, T.K., Kiever-Emmons, T., Blalick, J.E. and Kohler, H., Science, 240: 1034-1036, 1988). The insertion of self-binding peptide sequence into an antibody endows the property of self-binding and increases the valency and overall avidity for the antigen.

Similarly the addition of a signal peptide to antibodies facilitates transmembrane transport as demonstrate by Rojas et al, Nature Biotechnology, 16: 370-375 (1998). Rojas et al. have generated a fusion protein which contained a 12 mer peptide and have shown that this protein has cell membrane permeability.

Antibodies have been used as delivery devices for several biologically active molecules, such as toxins, drugs and cytokines. Often fragments of antibodies, Fab or scFv, are

preferred because of better tissue penetration and reduced "stickiness". Two methods to attach these molecules are known. One method is the design of a fusion gene and the expression of the fusion protein. This method requires extensive molecular biology engineering and depends typically on mammalian or bacterial expression systems in large scale fermentation. Another method of generating antibody complexes with other proteins or molecules relies on chemical cross-linking technologies. Usually hetero-bifunctional cross-linkers are used which cross-link the selected molecules at random sites on the immunoglobulin molecule. Hetero-bifunctional cross-linking is associated with two problems. First, the antibody structure is compromised by local protein denaturation at the sites of cross-linking. This leads to changes in half-life in blood and biodistribution and uptake by scavenger cells in lung and liver. The second problem is the potential loss of antigen binding by non-specific cross-linking to the antigen binding site.

A one chemical, site-specific cross-linking method exists (Rodwell, JD; Alvarez, VL; Lee, C; Lopes, AD; Goers, JW; King, HD; Powsner, HJ; McKearn, T., *Proc. Natl. Acad. Sci., USA,* 83:2632-6, 1986) which takes advantage of a unique carbohydrate site in the Fc domain of antibodies. This method has two disadvantages. First, cross-linking to the constant domain carbohydrate restricts this method to full-length antibodies and it cannot be used with variable domain fragments such as Fabs and scFvs. In addition, the method requires rather harsh chemical treatment using periodates for reducing the sugar to a reactive dialdehyde. This chemical reaction can damage other sensitive amino acid side chains, such as in tyrosine, in the Ig molecule leading to undesired changes in biodistribution or loss of antigen binding.

A variation of the carbohydrate site-specific cross-linking has been published by H. Hansen, et al., (Govindan, SV; Goldenberg, DM; Griffiths, GL; Leung, SO; Losman, MJ; Hansen, HJ., *Cancer Res.*, 55:5721-5725) who has introduced a

carbohydrate signal sequence by site-directed mutagenesis in the variable domain of an Ig light chain. This sequence allows the attachment of sugars to a serine residue during synthesis of the mutated antibody. The reducing chemistry to generate dialdehydes is then used with the above described pitfalls. In addition the site-directed insertion of a carbohydrate signal sequence requires molecular biology engineering and expression systems. Furthermore, the site of insertion in the Fv domain has to be carefully selected in order to avoid compromising antigen binding and/or stability of the heavy-light chain dimer structure.

Rajagopolan, et at., (*PNAS,* 93:6019-24, 1996) described the affinity site on antibodies for ATP and Adenosine. U.S. Patent 5,596,081 has issued for this site. A method of using azido-adensine and Azido-ATP has been described by Pavlinkova, et al., (*J. Immun. Methods,* 201:77-88,1997). The active binding peptide of C3d (complement fragment) has been described by Lambris, et al., (*PNAS,* 82:4235-39, 1985). The synthesis of 5-azido-tryptophan has been described by Miles & Phillips (Miles, E.W. & Phillips, R.S., *Biochemistry,* 24:4694-703, 1985). A method of photo-labeling is reviewed by Potter and Haley (Potter, R. & Haley, B.E., *Meth. Enzymol,* 91:6130633, 1982).

The affinity-site cross-linking chemistry of the present invention overcomes prior art problems in the art and does not require the molecular engineering steps and fusion protein expression, since it allows to cross-link selected peptides to full-length antibodies or antibody fragments in one step using mild photo-reactive chemistry.

SUMMARY OF THE INVENTION

The present invention provides a method of affinity cross-linking a peptide to an antibody comprising the steps of

(a) photo-chemically activating an azido compound in a peptide comprising said azido compound;

(b) adding an antibody to said photochemically activated

peptide; and

(c) allowing said photochemically activated peptide and said antibody to react, wherein said azido compound has an affinity for a hydrophobic structure in the variable domain of said antibody which binds to nucleotides or nucleosides, binding said peptide into a native binding pocket of the immunoglobulin (Ig) structure of an antibody, and wherein the site of cross-linking is located away from the antigen binding site in the Fv domain avoiding the compromise of antigen recognition.

In a preferred embodiment the photoreactive azido compound is created by oxidizing azido-adenosine with periodite to produce an azido-dialdehyde compound, wherein said dialdehyde is reacted via a Schiff-base reaction with primary amines of peptides of said antibody and photolyzed with UV light into an affinity site of said antibody.

In an alternative embodiment the azido compound is 5-azido tryptophan or 6-azido tryptophan, and wherein said azido compound is added to the C-terminal or N-terminal position of the peptide by standard peptide synthesis technology.

The invention also provides a composition and a pharmaceutical composition comprising a photochemically activated peptide having an N or C terminal azido compound, wherein said N or C terminal azido compound is crosslinked to an antibody and said azido compound has an affinity for a hydrophobic structure in the variable domain of said antibody which binds to nucleotides or nucleosides, binding said peptide into a native binding pocket of the immunoglobulin (Ig) structure of an antibody, and wherein the site of cross-linking is located away from the antigen binding site in the Fv domain avoiding the compromise of antigen recognition.

The invention of inserting biologically and immunologically active peptides into the variable domain of antibodies includes peptides which present T-cell and B-cell epitopes, comprise selfbinding, stimulate lymphocytes and allow transport across biological membranes.

The above and other objects of the invention will become readily apparent to those of skill in the relevant art from the following detailed description and figures, wherein only the preferred embodiments of the invention are shown and described, simply by way of illustration of the best mode of carrying out the invention. As is readily recognized the invention is capable of modifications within the skill of the relevant art without departing from the spirit and scope of the invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows that sera from mice immunized with C3d-3H1 were used in FACS of LS174T cells in a sandwich assay developed with FITC conjugated goat anti-mouse IgG. Control is a normal mouse serum. Cell number analyzed are plotted against relative fluorescence intensity on log10 scale.

Figure 2 shows two mice were immunized three times with 3H1 and two other mice with the 3H1-C3d peptide. Binding of sera dilutions of F(ab) of 3H1 as measured by ELISA is shown.

Figure 3 shows a tumor survival plot (Kaplan-Meier Plot) of mice pre-immunized with a) QS-1 (adjuvant), b) KLH-38C13 conjugate and QS-1 and 38C13-C3d peptide conjugate. Mice were immunized three times intraperional with 50 microgram conjugates or QS-1 solution. Mice were challenged with 38C13 tumor cells and survival was monitored. Ten mice were in each group.

Figure 4 shows that hybridoma BWZ.36 cells were co-cultured with purified mouse B-cells for 24 or 48 hrs. Response of T-cells is expressed as percentage of maximum response using x-gal assay. The percent (%) T-cell response is plotted against amount of peptide used. This result shows that affinity cross-linking of T-cell antigen peptide to antibodies which target presenting cells can significantly reduce the amount of effective peptide antigen and thereby reduce the need for immuno-stimulatory adjuvants.

Figure 5 shows Ig (OKT3 antibody) was first photolyzed with different concentrations of 6-azido-tryptophan followed by

photolysis with 8-azido-adenosine-biotin. Reaction mixtures were dot-blotted on cellulose membrane and developed with avidin-horse-radish-peroxidase and substrate. Color density of dot-blots were measured and are plotted against concentration of 6-azido-tryptophan.

DETAILED DESCRIPTION OF THE INVENTION

The present invention describes a method for chemically cross-linking peptides to an affinity site on antibodies. The affinity site on antibodies is highly conserved, consisting of framework residues within the variable domains of heavy and light chains (Rajagopalan, et al., *PNAS,* 93:6019-24, 1996). The affinity site binds heterocyclic structures like purine and tryptophan compounds. Cross-linking is achieved via an azido group introduced into, for example, adenosine, tryptophan or ATP. The use of a photo-reactive azido compound for affinity cross-linking has been demonstrated for affinity cross-linking of biotin to an antibody (Pavlinkova, et al., *J. Immun. Methods,* 201:77-88, 1997).

The affinity-site cross-linking chemistry of the present invention overcomes prior art problems in the art and does not require the detailed molecular engineering and fusion protein expression steps, since it allows the cross-linking of any selected peptide to full-length antibodies or antibody fragments in one step using mild photo-reactive chemistry.

The site of cross-linking is located away from the antigen binding site in Fv domain avoiding compromising antigen recognition. Furthermore, cross-linking occurs into a native binding pocket while the overall Ig structure is kept intact. It is clear that this chemical method of cross-linking a peptide to an antibody is cost-saving and significantly less labor intensive than a molecular biology approach.

Thus, the present invention provides a method of affinity cross-linking a peptide to an antibody comprising the steps of

(a) photo-chemically activating an azido compound in a peptide comprising said azido compound;

(b) adding an antibody to said photochemically activated peptide; and

(c) allowing said photochemically activated peptide and said antibody to react, wherein said azido compound has an affinity for a hydrophobic structure in the variable domain of said antibody which binds to nucleotides or nucleosides, binding said peptide into a native binding pocket of the immunoglobulin (Ig) structure of an antibody, and wherein the site of cross-linking is located away from the antigen binding site in the Fv domain avoiding the compromise of antigen recognition. Each antibody has two variable domain structures and two molecules of peptides may be photo-inserted into one molecule of antibody.

The method of the invention may, further comprise a step of stabilizing the cross-linking with a mild reducing reagent. In a preferred embodiment the mild reducing agent is borohydrate.

The peptide of the invention may have a biological activity, such as immuno-stimulatory or immuno-regulatory activity. In a preferred embodiment the peptide is derived from the binding site region of cytokines or complement fragments. The peptide may comprise immunogenic epitopes for T-cells or B-cells. The peptide may, for example, be a hormone, ligand for cytokines or a binding site derived from natural ligands for cellular receptors. In a preferred embodiment the peptide is derived from C3d region 1217-1232 and ranges from about 10 to about 16 mer. In an alternative embodiment the peptide is a 16mer azido-peptide derived from the C3d region 1217-1232. The peptide may comprise 8-azido-adenosine attached to alpha or epsilon primary amine, 5-azido-tryptophan or 6-azido-tryptophan. The peptide may be bound to an antibody which is a full-length immunoglobulin molecule or a variable domain fragment of an antibody. The antibody is preferably specific for a cellular receptor, on a membrane structure such as a protein, glycoprotein,

polysaccharide or carbohydrate, and on a normal cell or on tumor cells.

In the method of the invention the photo-chemical reaction may be mediated by a photo chemical compound selected from azido derivatized adenosine, ATP and tryptophan. The photo-chemical compound has an affinity for a hydrophobic structure in the variable domain of antibodies which binds to nucleotides or nucleosides.

The use of peptides derived from the ligand site of C3d as an immunostimulatory component incorporated into antibodies has an unexpected utility as a molecular adjuvant. C3d has been used as molecular adjuvant as part of a complete fusion protein with hen egg lysozyme (HEL) by D. Fearon, et al., (Dempsey, P.W., Allison, M.E.D., Akkaraju, S., Goodnow, C.C. and Fearon, D.T., *Science,* 271:348, 1996). These authors have shown that a HEL- C3d fusion protein is up to 10,000 fold more immunogenic than free HEL (see International Patent Publication, WO96/17625).

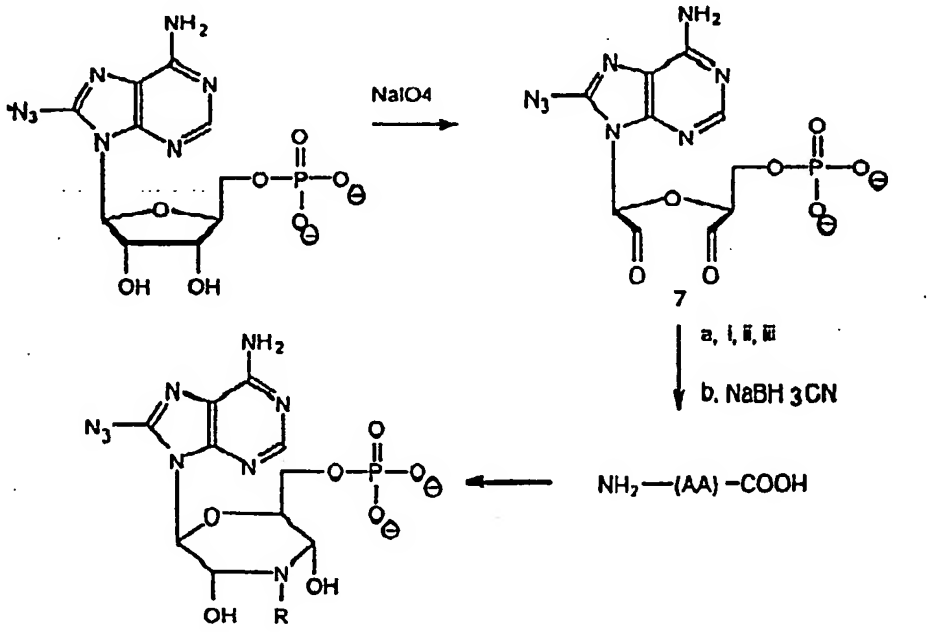
Similar increases in immunogenicity have been observed with chemical cross-linked idiotype vaccines using a peptide derived from the C3d fragment in our recent animal studies (see examples below). It is believed that attaching C3d peptides to idiotype and anti-idiotype vaccines enhances the immunogenicity of these vaccines and substitutes for the need of attaching carrier molecules such as KLH in combination with strong adjuvants such Freund adjuvant which is not permitted by the FDA for humans. In an alternative embodiment the peptide may be derived from a human or non-human C3d region homologous to the human C3d residues at position 1217-1232 and ranges from about 10 to about 16 mer.

Other applications of affinity cross-linking biologically active peptides to antibody vaccines include active peptides derived from cytokines. For example, a nonapeptide from the IL1-beta cytokine has been described (Antoni, et al., *J. Immunol,* 137:3201-04, 1986) which has immunostimulatory

properties without inducing undesired side effects. Other examples of active peptides which can be inserted into antibodies in accordance with the invention include signal peptides, antigenic peptides, and peptides from the selfbinding locus of antibodies.

Two methods are used to affinity cross-link peptides to antibodies. In the first method azido-adenosine is oxidized with periodite to produce azido-adenosine-dialdehyde. The dialdehyde is reacted via a Schiff-base reaction to the primary amines of peptides (alpha and epsilon amino groups). The chemical bound is stabilized by a mild reducing reagents, such as borohydrate (see scheme I).

Scheme I



The azido-adenosine-peptide is photolyzed with UV light into the affinity site of antibodies. Since each antibody has two variable domain structures, two molecules of peptides can be photo-inserted into one molecule of antibody. 6-Azido-L-tryptophan is synthesized according to Miles and Phillips (Miles, E.W. & Phillips, R.S., *Biochemistry* 24: 4694-703,

1985). A variety of peptides are known having biological activities as hormones, ligands for cytokines or binding sites derived from natural ligands for cellular receptors. Also peptides have been recognized which represent the major antigenic determinants of proteins for stimulating B-cells to produce antibodies or for T-cells to generate helper of cytolytic T- cells. These peptides can be targeted to selected cellular targets via complexes consisting of affinity-cross-linked antibodies against specific cellular receptors on normal cells or to tumor cells. By targeting active peptides to specific targets biological or immunological processes can be induced without systemic side effects due to non-specific systemic deliveries.

For example, the present inventors have attached peptides, which represents the active binding region of complement to complement receptors on B-cells, to an antibody idiotype vaccine. This peptide-antibody complex increased the immunogenicity of the anti-idiotype vaccine, simulating the adjuvant effect of Freund's adjuvant or conjugation to a carrier protein, such as keyhole lympet hemocyanin (KLH).

The method of making and using the affinity-cross-linking C3d derived peptides has been demonstrated in two systems in animal experiments.

EXAMPLE 1

Enhancement of an anti-idiotype vaccine.

3H1 is a murine anti-idiotype antibody (Bhattacharya-Chatterjee, et al., *J. Immunol.*, 145:2758-65, 1990) which mimics the carcino-embryonic antigen (CEA). 3H1 induces in animals anti-CEA antibodies when used as KLH-conjugated vaccine in complete Freund's adjuvant. 3H1 has also been tested in a clinical phase I study where it induces antibodies which bind to CEA in approximately half of treated cancer patients. However no clinical response was observed in this study (Foon, et al., *J. Clin. Invst.*, 96:334-342, 1995) due, in part, to low

immunogenicity.

3H1 mAb was affinity cross-linked with a 13mer peptide derived from the C3d region 1217-1232. The amino acid sequence was derived from of the Cd3 peptide -KNRWEDPGKOLYNVEA-. BALB/c mice were immunized twice with 25 ug of C3d-3H1 in phosphate-saline solution intramuscular. 7 days after the last immunization mice were bled and sera were tested for binding to 8019 (Ab1 idiotype) and to the CEA expressing tumor line LS174T. As seen in figure 1 sera from C3d-3H1 immune mice bind to LS174T tumor cells as determined in FACS, while a control serum (normal mouse serum) showed only background fluorescence.

Sera from mice immunized with C3d-3H1 were used in FACS of LS174T cells in a sandwich assay developed with FITC conjugated goat anti-mouse IgG (Fig. 1). Control is a normal mouse serum. Cell number analyzed are plotted against relative fluorescence intensity on log10 scale.

EXAMPLE 2

Furthermore, sera from mice immunized three times with either 3H1 (25 microgram in saline) or 3H1-C3d-peptide (affinity cross-linked, 25 microgram in saline) were also tested for Ab3 response. Mice were bled and sera were tested for binding to F(ab) of 3H1 in ELISA. Figure 2 shows the binding of dilutions of mouse sera to 3H1 F(ab). While nacked 3H1 does not induce Ab3 antibodies, 3H1-peptide does showing that the affinity-cross-linked 3H1 enhanced immunogenicity. Other C3d peptides which may be used in the practice of the present invention include those reviewed in Lambris et al, "Phylogeny of the third component of complement, C3" in Erfi, A ed. New Aspects of Complement structure and function, Austin, R.D. Landes Co., 1994 p. 15-34, incorporated herein by reference in its entirety.

EXAMPLE 3

Enhancement of an mouse Tumor Idiotype Vaccine (38C13).

38C13 is the idiotype expressed by the 38C13 B-lymphoma

tumor cell line. The Levy group has developed this idiotype tumor vaccine model and has shown that pre-immunization with KLH-conjugated 38C13 Id can protect against challenge with 38C13 tumor cells in mice (Kaminski, M.S., Kitamura, K., Maloney, D.G. and Levy, R., *J. Immunol,* 138:1289, 1987). Levy and colleagues (Tao, M-H. and Levy, R., *Nature* 362:755-758, 1993) have also reported on the induction of tumor protection using a fusion protein (CSF-38C13), generated from a chimeric gene and expressed in mammalian cell culture fermentation. 38C13 Id proteins was affinity cross-linked with a 16mer azido-peptide derived from the C3D region 1217-1232.

10 mice were immunized with 50ug of C3d-38C13 conjugate in phosphate-saline solution intraperitoneally three times. After the third vaccination mice were challenge with 38C13 tumor cells. Control groups included mice vaccinated with 38C13-KLH in QS-21 (adjuvant), considered the "gold standard" in this tumor model, and mice injected with QS-21 alone. As seen in Figure 3, 7 out 10 mice vaccinated with the C3d-38C13 conjugate survived by day 35 after tumor challenge, as did mice vaccinated with the KLH-38C13 in QS-21. All control mice injected only with QS-21 had died by day 22.

C3H mice were immunized three times with either 38C13-KLH in QS-21 or with 38C13-C3d peptide without QS-21 (50 ug i.p.) Control mice were only injected with QS-21. Immunized and control mice were than challenged with 38C13 tumor cells and survival was monitored (Fig. 3).

Results illustrated in Examples 1 - 3 show that affinity-cross-linking of an immuno-stimulatory peptide to tumor anti-idiotype and idiotype vaccine antibodies can significantly enhance the immune response to the tumor and protect against tumor challenge. The vaccination protocol with the C3d-cross-linked vaccine did not include any adjuvant, such as Freund's adjuvant, or KLH conjugation, both of which are not permissible by the FDA for human use.

EXAMPLE 4

Enhanced antigen presentation by an affinity cross-linked T-cell epitope peptide.

The peptide derived from the sequence 323-339 of ovalbumin (OVA) is one of the major T-cell epitopes in mice. A mouse T-cell hybridoma BWZ.36 clone responds with activation of beta-gal producing color with x-gal substrate when challenged with the T-cell peptide in vitro in the presence of presenting B-cells. The dose of OVA peptide for maximum response is in the 5-10 microgram/ml. In order to improve presentation of the peptide to T-cells the ability of anti-Ig antibodies to target the peptide to the presenting B-cells was enhanced. This was achieved by affinity-cross-linking the peptide to an anti-Fab antibody. Peptide-conjugated antibody induced maximum response at a peptide concentration of 0.01ug/ml. Thus T-cell epitope peptide presented by anti-Ig to B-cells reduces the peptide dose more than 1000 fold. It is believed that the peptide-anti-Fab binds to the B-cell receptor on B-cells and is internalized induced by cross-linking the Ig receptor followed by uptake of the receptor antibody complex.

The following cultures were assayed: 1. Peptide, presenting B-cells were incubated with T-cell peptide at indicated concentration; peptide and Ig (50 ug), peptide together with 50 microgram of normal mouse IgG; Fab-peptide, anti-Fab IgM affinity-cross-linked with T-cell peptide; Fab-peptide and Ig50ug, anti-Fab IgM affinity-cross-linked with T-cell peptide together with 50 microgram of normal mouse IgG; peptide and Ig10ug, peptide together with 10 microgram of normal mouse IgG; Fab-peptide and Ig10ug, anti-Fab IgM affinity-cross-linked with T-cell peptide together with 10 microgram of normal mouse IgG.

EXAMPLE 5

Azido-tryptophan is shown to compete with the nucleoside affinity-photobiotinylation. In order to demonstrate that azido-tryptophan containing peptides can be cross-linked to the

nucleoside affinity site competition photobiotinylation experiments were performed. As seen in Figure 5, 6-azido-tryptophan competes with 8-azido-adenosone-biotin effectively.

Some of the procedures used in the above examples are known; for more detail see, a method of using azido-adenosine and Azido-ATP has been described by Pavlinkova, et al., (*J. Immun. Methods,* 201:77-88, 1997). The active binding peptide of C3d (complement fragment) has been described by Lambris, et al., (*PNAS,* 82:4235-39, 1985) and is incorporated herein by reference in its entirety. The synthesis of 5-azido-tryptophan has been described by Miles & Phillips (Miles, E.W. & Phillips, R.S., *Biochemistry* 24:4694-703, 1985).

As seen in Figure 4, the amount of T-cell peptide needed in the in vitro T-cell response is more than two logs lower, compared to added free peptide, if the peptide is conjugated to the anti-Fab antibody which delivers the peptide to the presenting B-cells. Hybridoma BWZ.36 cells were co-cultured with purified mouse B-cells for 24 or 48 hrs.

Prior to this culture B-cells had been exposed to goat ant-Fab IgM, mixtures of peptide and ant-Fab IgM and ant-Fab IgM-peptide complex. Response of T-cells is expressed as percentage of maximum response using x-gal assay. % T-cell response is plotted against amount of peptide used.

This result shows that affinity cross-linking of T-cell antigen peptide to antibodies which target presenting cells can significantly reduce the amount of effective peptide antigen and thereby, advantageously and unexpectedly reduces the need for immuno-stimulatory adjuvants. While non-site-specific peptide conjugation to targeting antibodies may also induce similar immuno-enhancing effects, the affinity-site specific cross-linking preserves the critical antigen binding site of targeting and delivering antibodies. This property represents an advantage over conventional cross-linking methods by

reducing the effective dose of peptide-conjugated antibody preparation.

The results illustrated in Examples 1 to 5 above, demonstrate that affinity-cross-linking of immunologically active peptides to antibodies can result in enhancement of immunogenicity of idiotype antibody vaccines and improvement of peptide presentation to T-cells. These advantages are useful in the design of novel and more efficient anti-tumor or anti-microbe vaccines for treating human diseases. The key to such improved prophylactic and therapeutic vaccines is the unique method of affinity-cross-linking peptides into a site on antibodies which does not interfere with antigen binding and preserves the structural and functional integrity of the antibody molecule.

The invention also provides a composition and a pharmaceutical composition comprising a photochemically activated peptide having an N or C terminal azido compound, wherein said N or C terminal azido compound is crosslinked to an antibody and said azido compound has an affinity for a hydrophobic structure in the variable domain of said antibody which binds to nucleotides or nucleosides, binding said peptide into a native binding pocket of the immunoglobulin (Ig) structure of an antibody, and wherein the site of cross-linking is located away from the antigen binding site in the Fv domain avoiding the compromise of antigen recognition.

Any antibody may be used in the peptide/antibody complex of the invention. Preferred antibodies are anti-idiotype antibodies. For example, anti-idiotype antibody 3H1 may be used (see "Anti-idiotype Antibody Vaccine (3H1) that Mimics the Carcinoembryonic Antigen (CEA) as an Adjuvant Treatment", Foon, et al., *Cancer Weekly*, June 24, 1996). Other anti-idiotype antibodies which may be used in the present invention include, for example, anti-idiotype antibody to chlamydia glycolipid exoantigen (U.S. Patent No. 5,656,271; anti-idiotype antibody 1A7 for the treatment of melanoma and small cell carcinoma (U.S. Patent No. 5,612,030); anti-idiotype antibody MK2-23

anti-melanoma antibody (U.S. Patent No. 5,493,009); anti-idiotypic gonococcal antibody (U.S. Patent No. 5,476,784) Pseudomonas aeruginosa anti-idiotype antibody (U.S. Patent No. 5,233,024); antibody against surface Ig of Human B cell tumor (U.S. Patent No. 4,513,088); and monoclonal antibody BR96 (U.S. Patent No. 5,491,088). In a preferred embodiment the molar concentration of the cross-linking peptide is about 150 micro Moles. Any restrictions on peptide length are those practical limitations associated with peptide synthesis and not restrictions associated with practice of the method of the invention.

In an alternative embodiment, self-binding peptides such as those disclosed in (Kang, C.Y. Brunck, T.K., Kiever-Emmons, T., Blalick, J.E. and Kohler, H., "Inhibition of self-binding proteins (auto-antibodies) by a VH-derived peptide, Science, 240: 1034-1036, 1988, incorporated herein by reference in its entirety) used in the method of the present invention.

Additionally signal peptides such as those disclosed in Roias, et al., "Genetic Engineering of proteins with cell membrane permeability", Nature Biotechnology, 16: 370-375 (1988) and Calbiochem Signal Transduction Catalogue 1997/98, incorporated herein by reference in their entireties, may be used in the method of the invention.

Moreover, antigenic peptides such as those disclosed in Pincus et al, "Peptides that mimic the group B streptococcal type II capsular polysaccharide antigen", J. Immunol., Vol 160: 293-298 (1998) [incorporated herein by reference in its entirety] may be substituted for the C3d peptide in example 1, and may be used in the practice of the present invention.

In a preferred embodiment the antibody is crosslinked to a peptide is which is a photoreactive benzophenon-derivative of phenylalanine, such as p-benzoyl-DL-phenylalanine. The photoreactive benzophenon-derivative of phenylalanine is added to the C-terminal or N-terminal position of said peptide.

The peptide may be designed to have inverse hydropathic character and exhibits mutual affinity and homophilic (self)

binding within the peptide, in accordance with the disclosure of United States Patent No. 5,523,208 (incorporated herein by reference in its entirety).

The invention also encompasses a composition comprising an antibody crosslinked to a peptide produced by expressing a DNA encoding a fusion protein comprising the peptide and either the light or heavy chains of the antibody. The fusion protein may be prepared by the method disclosed in "Molecular Cloning: A Laboratory Manual, Second Ed., Cold Spring Harbor Press, 1989 (incorporated herein by reference in its entirety).

The compositions of the invention are useful in pharmaceutical compositions for systemic administration to humans and animals in unit dosage forms, sterile solutions or suspensions, sterile non-parenteral solutions or suspensions oral solutions or suspensions, oil in water or water in oil emulsions and the like, containing suitable quantities of an active ingredient. Topical application can be in the form of ointments, creams, lotions, jellies, sprays, douches, and the like. The compositions are useful in pharmaceutical compositions (wt%) of the active ingredient with a carrier or vehicle in the composition in about 1 to 20% and preferably about 5 to 15%.

The above parenteral solutions or suspensions may be administered transdermally and, if desired a more concentrated slow release form may be administered. The cross-linked peptides of the invention may be administered intravenously, intramuscularly, intraperitoneally or topically. Accordingly, incorporation of the active compounds in a slow release matrix may be implemented for administering transdermally. The pharmaceutical carriers acceptable for the purpose of this invention are the art known carriers that do not adversely affect the drug, the host, or the material comprising the drug delivery device. The carrier may also contain adjuvants such as preserving stabilizing, wetting, emulsifying agents and the like together with the penetration enhancer of this invention.

The effective dosage for mammals may vary due to such factors as age, weight activity level or condition of the subject being treated. Typically, an effective dosage of a compound according to the present invention is about 10 to 500mg, preferably 2-15 mg, when administered by suspension at least once daily. Administration may be repeated at suitable intervals.

The purpose of the above description and examples is to illustrate some embodiments of the present invention without implying any limitation. It will be apparent to those of skill in the art that various modifications and variations may be made to the composition and method of the present invention without departing from the spirit or scope of the invention. All patents and publications cited herein are incorporated by reference in their entireties.

What is Claimed is:

1. A method of affinity cross-linking a peptide to an antibody comprising the step of

photochemically activating an azido compound in a peptide comprising said azido compound;

adding an antibody to said photochemically activated peptide; and

allowing said photochemically activated peptide and said antibody to react, wherein said azido compound has an affinity for a hydrophobic structure in the variable domain of said antibody which binds to nucleotides or nucleosides, binding said peptide into a native binding pocket of the immunoglobulin (Ig) structure of an antibody, and wherein the site of cross-linking is located away from the antigen binding site in the Fv domain avoiding the compromise of antigen recognition.

2. The method of claim 1, wherein said photoreactive azido compound is created by oxidizing azido-adenosine with periodite to produce an azido-dialdehyde compound, wherein said dialdehyde is reacted via a Schiff-base reaction with primary amines of peptides of said antibody and photolyzed with UV light into an affinity site of said antibody.

3. The method of claim 1, wherein said azido compound is 5-azido tryptophan or 6-azido tryptophan, and wherein said azido compound is added to the C-terminal or N-terminal position of the peptide by standard peptide synthesis technology.

4. The method of claim 1, wherein each antibody has two variable domain structures and two molecules of peptides are photo-inserted into one molecule of antibody.

5. The method of claim 1, further comprising a step of stabilizing the cross-linking with a mild reducing reagent.

6. The method of claim 1, wherein the mild reducing agent is borohydrate.

7. The method of claim 1, wherein said peptide is derived from a human or non-human C3d region homologous to the human C3d residues at position 1217-1232 and ranges from about 10 to about 16 mer.

8. The method of claim 1, wherein said peptide is a 16mer azido-peptide derived from the a human or non-human C3d region homologous to the human C3d residues at position 1217-1232.

9. The method of claim 1, wherein said peptide comprises 8-azido-adenosine attached to alpha or epsilon primary amines.

10. The method of claim 1, wherein said peptide comprises 5-azido-tryptophan or 6-azido-tryptophan.

11. The method of claim 1, wherein said peptide has a biological activity selected from the group consisting of immuno-stimulatory, membrane transport, homophilic and antigenic activities.

12. The method of claim 1, wherein said peptide is derived from the binding site region of cytokines or complement fragments.

13. The method of claim 1, wherein said peptide comprises immunogenic epitopes for T-cells or B-cells.

14. The method of claim 1, wherein said antibody is specific for a cellular receptor, or a membrane structure on a normal cell or on tumor cells.

15. The method of claim 1, wherein said antibody is a full-length immunoglobulin molecule or a variable domain fragment of an antibody.

16. The method of claim 1, wherein the photochemical reaction is mediated by a photochemical compound selected from the group consisting of azido derivatized adenosine, ATP and tryptophan and other heterocyclic compounds.

17. The method of claim 12, wherein said photochemical compound has an affinity for a hydrophobic structure in the variable domain of antibodies which binds to nucleotides or nucleosides.

18. The method of claim 1, wherein said peptide is selected from the group consisting of hormones, ligands for cytokines and binding sites derived from natural ligands for cellular receptors.

19. The method of claim 1, wherein the crosslinking peptide is a photoreactive benzophenon-derivative of phenylalanine and wherein said photoreactive benzophenon-derivative of phenylalanine is added to the C-terminal or N-terminal position of said peptide.

20. The method of claim 19, wherein the photoreactive benzophenon-derivative of phenylalanine is p-benzoyl-DL-phenylalanine.

21. The method of claim 1, wherein said peptide is designed to have inverse hydropathic character and said peptide exhibits mutual affinity and homophilic binding, within the length of said peptide.

22. A composition comprising a photochemically activated peptide having an N or C terminal azido compound, wherein said N or C terminal azido compound is crosslinked to an antibody and said azido compound has an affinity for a hydrophobic structure in the variable domain of said antibody which binds to nucleotides or nucleosides, binding said peptide into a native binding pocket of the immunoglobulin (Ig) structure of an antibody, and wherein the site of cross-linking is located away from the antigen binding site in the Fv domain avoiding the compromise of antigen recognition.

23. The composition of claim 22, wherein said azido compound is 5-azido tryptophan or 6-azido tryptophan, and wherein said azido compound is added to the C-terminal or N-terminal position of the peptide by standard peptide synthesis technology.

24. The composition of claim 22, wherein said antibody is specific for a cellular receptor on a normal cell or on tumor cells.

25. The composition of claim 22, wherein said antibody is a full-length immunoglobulin molecule or a variable domain fragment of an antibody.

26. A composition comprising an antibody crosslinked to a peptide having inverse hydropathicity within the length of said peptide.

27. A composition comprising an antibody crosslinked to a peptide having a biological activity selected from the group consisting of immuno-stimulatory, membrane transport, homophilic and antigenic activities.

28. A composition comprising an antibody crosslinked to a peptide produced by expressing a DNA encoding a fusion protein comprising said peptide and either the light or heavy chains of said antibody.

FIG. 1

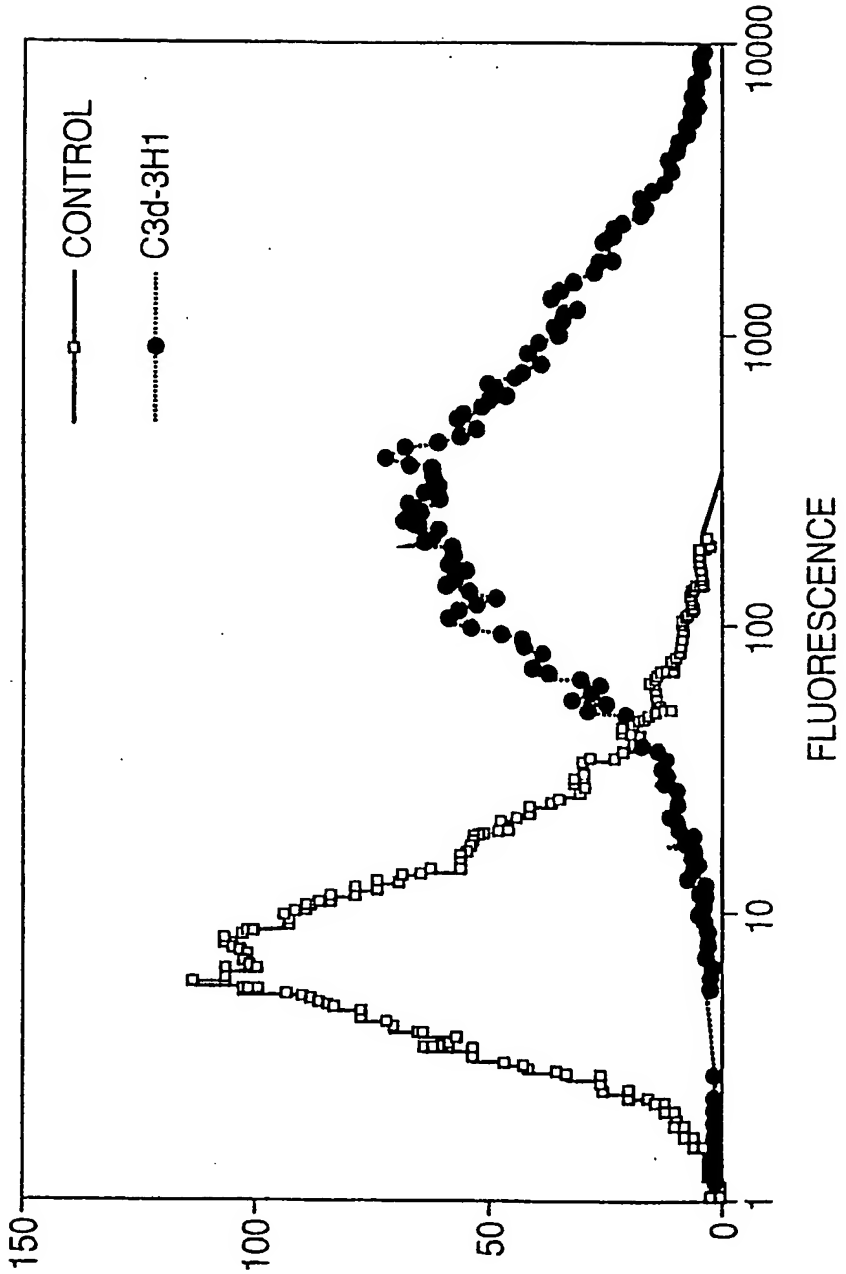
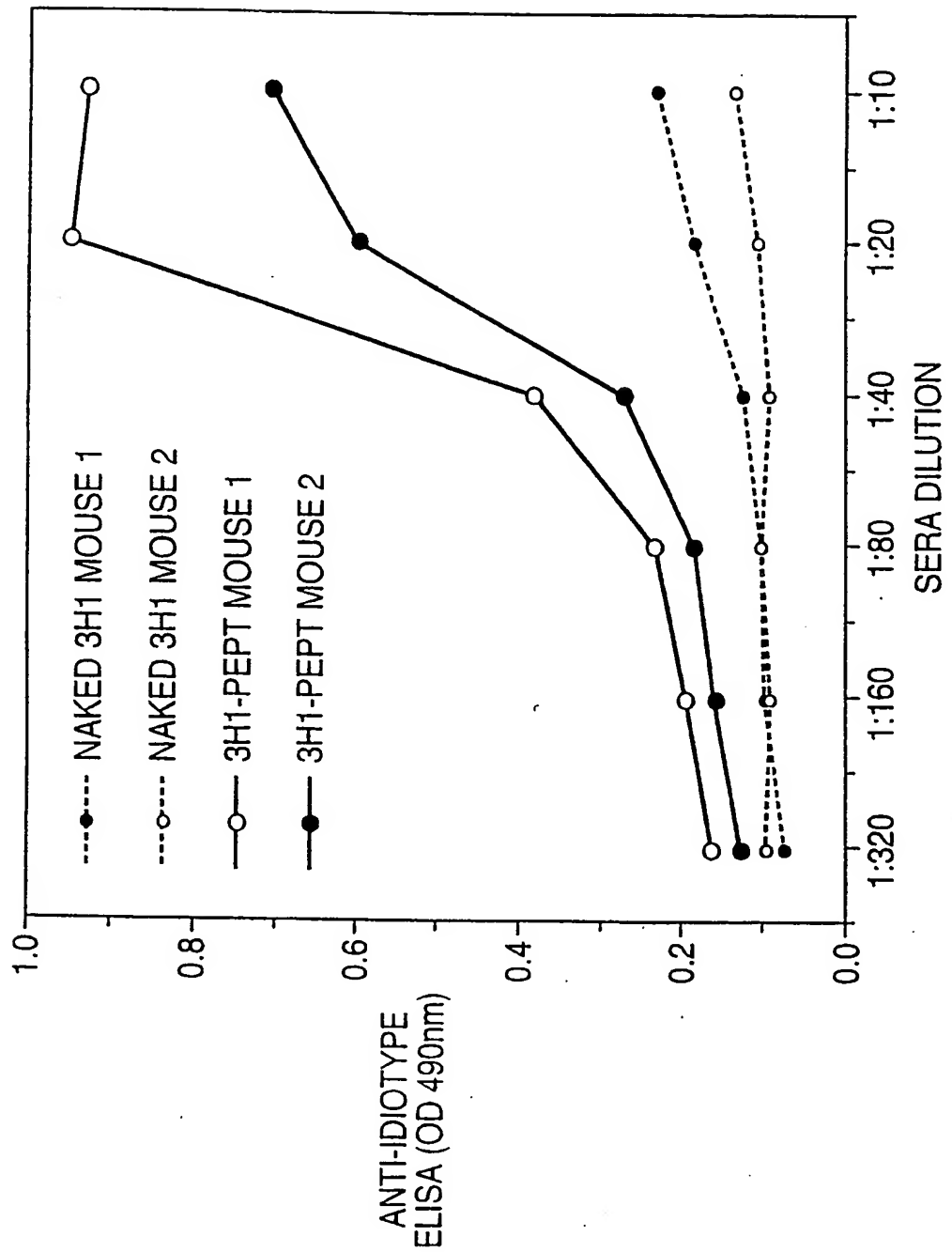


FIG. 2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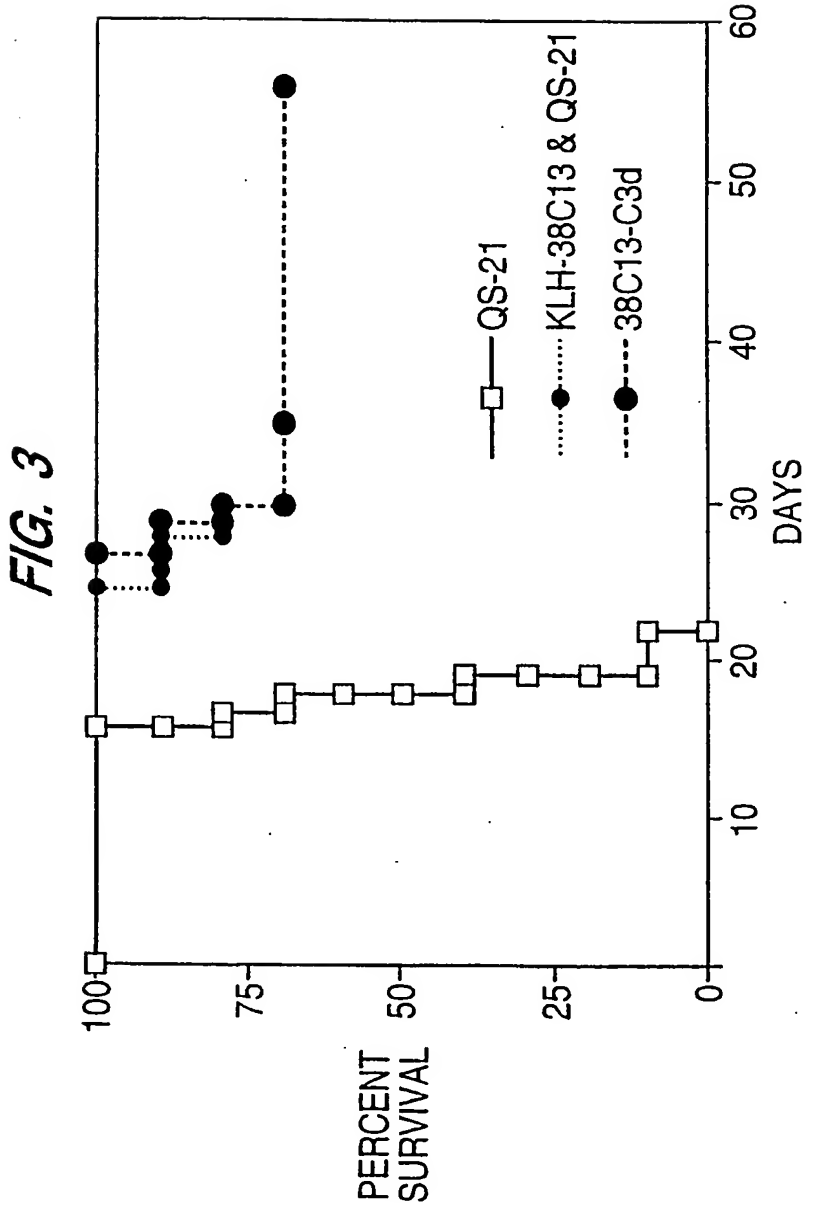
SERA DILUTION

FIG. 3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KLH-38C13 & QS-21
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FIG. 4

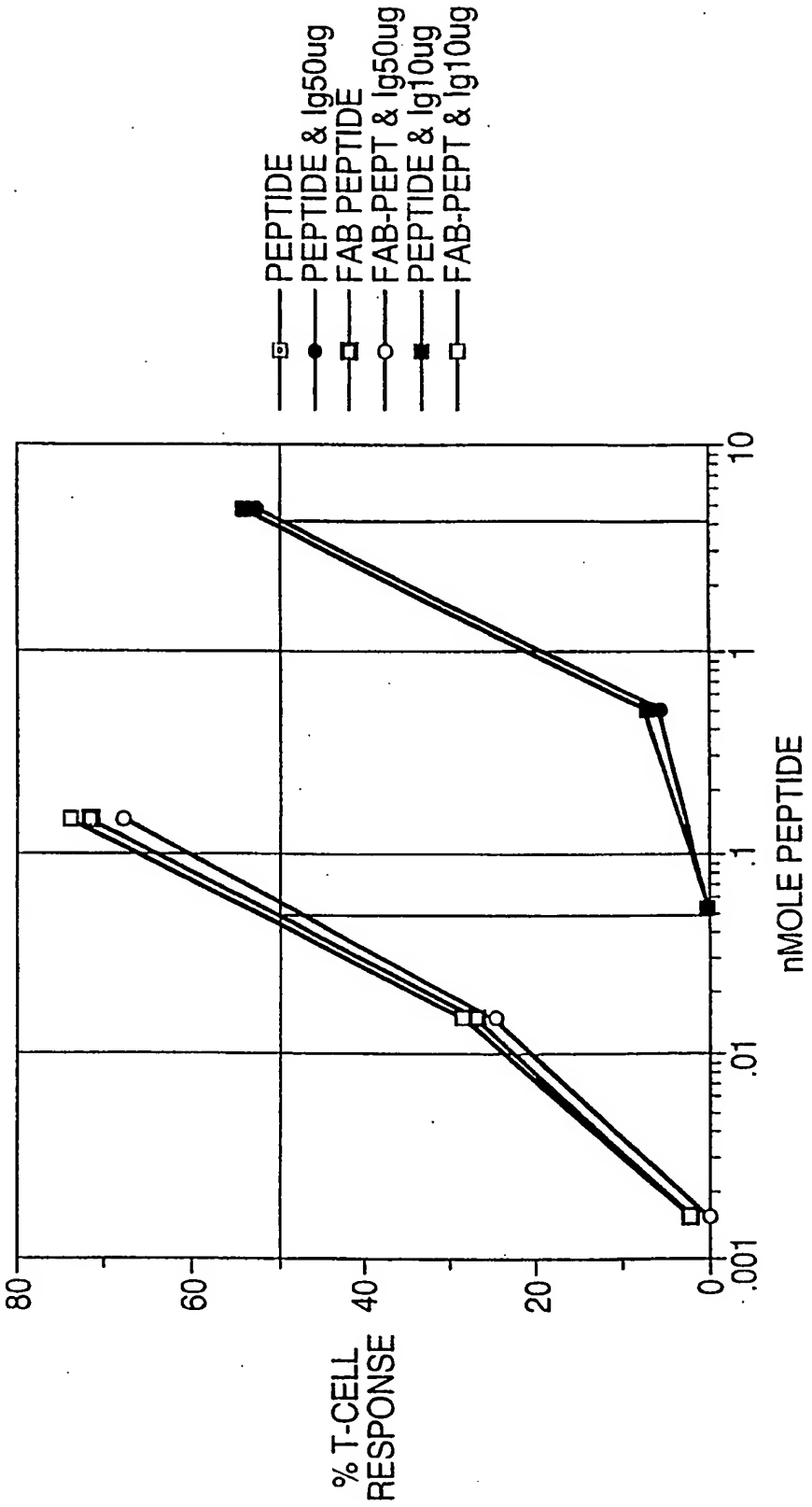
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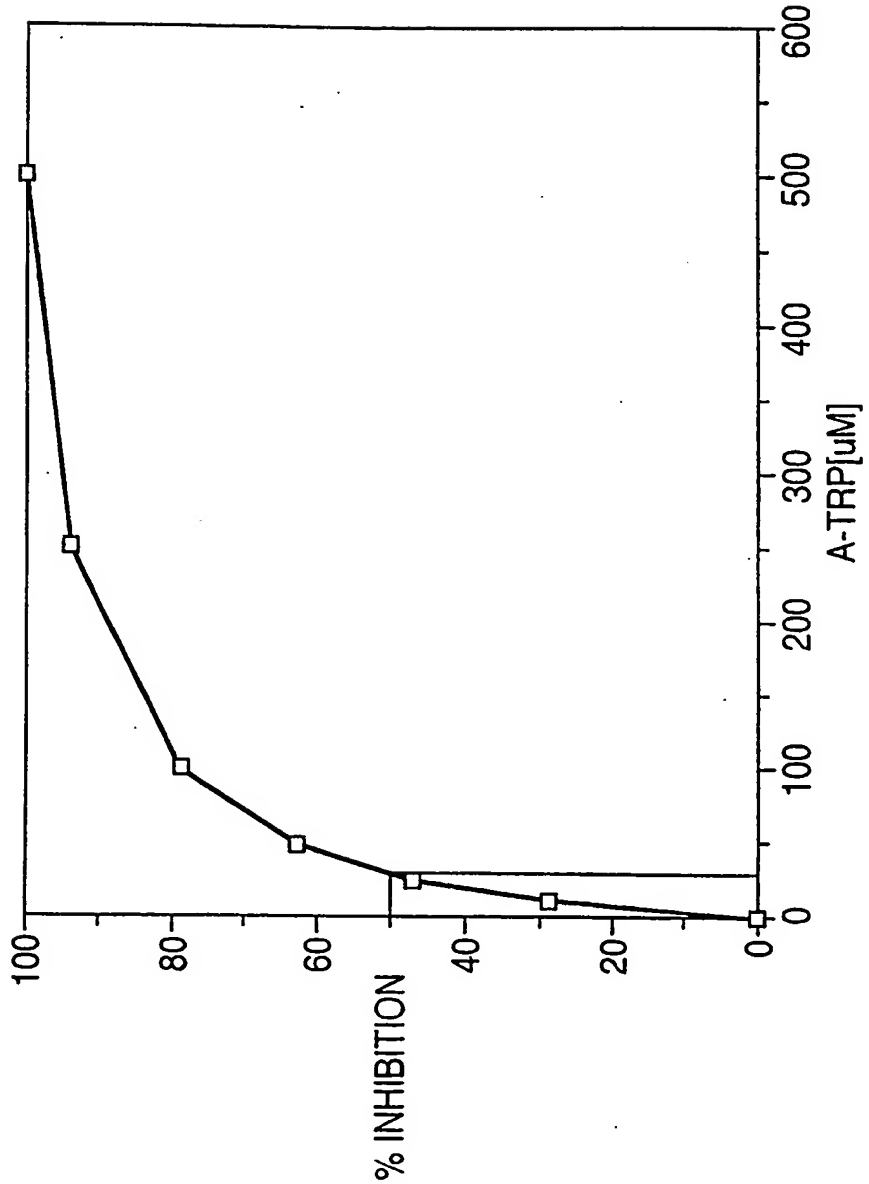
FIG. 5



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/19710

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) : C07K 16/00; C12P 21/00
US CL : 530/391.3, 391.1; 436/547
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/391.3, 391.1; 436/547

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,596,081 A (HALEY et al) 21 January 1997, col. 16, lines 23-38.	1-4, 9-10, 14-17, 19-22
X, P	US 5,800,991 A (HALEY et al) 01 September 1998, col. 7, lines 44-55; col. 8, lines 61-65; col. 10, lines 30-44; col. 11, lines 10-44; col. 12, line 62 to col. 13, line 4; col. 18, lines 1-7; col. 14, Example 11; col. 25, Example 13 to col.27, line 12.	1-4, 9-10, 16-17, 19-22
Y	US 5,276,170 A (KIRK et al) 04 January 1994, col. 4, lines 23-43; col. 5, line 17 to col. 6, line 39; col. 8, lines 21-31.	1, 19

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
06 DECEMBER 1998

Date of mailing of the international search report
20 JAN 1999

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/19710

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PANDEY, R. et al. Photochemical linking of primary aromatic amines to carrier proteins to elicit antibody response against the amine haptens. Journal of Immunological Methods. (1986). Vol. 94, pages 237-246, entire document.	1-22
Y	LAMABRIS, J.D. et al. Mapping of the C3d receptor(CR2)-binding site and a neoantignic site in the C3d domain of the third component of complement. Proc. Natl. Aca. Sci. USA. June 1985, Vol. 82, pages 4235-4239, especially, page 4235.	7-8, 13